Notch ligand functionalized microbead system for T cell differentiation.

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ABSTRACT

Hematopoietic stem cells (HSC) are cells that can self-replicate and give rise to a wide array of different cell types through the process of differentiation. HSC are found in blood and bone marrow, and they are responsible for the daily production of blood and immune cells essential to humans' survival. HSCs' multipotency makes it a powerful candidate for new, revolutionary treatments for diseases. However, in order to harness its multpotency, we must first understand what instructs HSC to become very specific cell types. In particular, we are interested in the role of the Notch signaling pathway in HSC differentiation into T-cells, white blood cells that regulate the immune system and destroy tumor and virus-infected cells. The Notch signaling pathway been shown to be necessary and sufficient for directing HSC differentiation into T-cells. Specifically we limit our studies to two proteins in the pathway: Notch1 receptor on the signal receiving cell's membrane and delta-like ligand 4, a protein that activates Notch1. However, scalable and efficient biomaterial-based systems for this differentiation process have yet to be reported. Such a system would allow unprecedented temporal and ligand density control, prerequisites for the ultimate goal of mass producing T-cells "on demand" for therapeutic treatments for immunodeficient or cancer patients. Here we describe an artificial Notch signaling system using magnetic microbeads coated with DLL4 to activate Notch1 receptors.

The DLL4 have been shown to be capable of directing HSC differentiation to T cells. The aim of my project is to characterize the cell-bead interaction to better understand how Notch1 activation varies with DLL4 density and temporal presentation. Mouse embryonic stem cells and myoblasts (muscle progenitor cells) were used as model cell lines since HSCs are difficult to maintain in culture. Although RT-PCR results indicated that DLL4 beads induced a

3-5 fold increase in HES1 (a downstream target gene of Notch1 signaling) gene expression, fluorescent labeling of activated Notch1 was unable to distinguish basal level of Notch activity with activation induced by the DLL4 beads. Although my studies were not able to confirm DLL4 bead activation of Notch1 with fluorescent labeling, the results will aid in developing the parameters for using murine ES cells and myoblasts as model systems to test the functionality of beads and bead-cell interaction. Knowledge gained from this study and the proposed future experiments will enhance our understanding of *in vitro* T cell generation. Moreover, continuation of the cell-bead interaction studies would further contribute to the development of a biomaterials-based system for high throughput T cell production for novel therapies.

INTRODUCTION

This project is part of a long-term goal to develop a biosynthetic system for efficient and high throughput production of T cells (white blood cells that are essential for a functional immune system) from hematopoietic stem cells (HSCs), progenitor cells that can give rise to all blood cell types. My role is to characterize and optimize the use of a synthetic signaling substrate (protein-coated magnetic beads) to activate an important cell signaling pathway called Notch. Although use of protein-coated beads is common, the Roy lab pioneered the use of these beads to direct major cell developmental processes. (pioneered is kind of a strong word, he's developed a novel system for ex vivo differentiation, but when you use pioneer it makes it seem like we're the first ones to do this, maybe find another word?) I aimed to evaluate different parameters of the bead interaction with signal receiving cells in order to optimize this biomaterial system, which is an artificial niche created with both synthetic and natural components. Specifically, I stained proteins in the Notch pathway with fluorescent molecules and examined Notch gene expression to examine the relationship between bead density and incubation time on Notch activation. The goal was to optimize the bead-cell interaction in the biomaterial system for the efficient production of T cells.

One may ask why such a system for the T cell development would be necessary. As will explained in detail below, T cells recognize and eliminate diseased or tumor cells, thereby preserving the health and survival of the human. Lack or destruction of T cells dramatically decreases a human's ability to survive. For example, HIV targets and destroys T cells, leaving AIDS patients unable to combat infections, so patients eventually succumb to death. However, if scientists could harness and control the self-replicating ability and plasticity of HSC, novel and effective medical treatments may be developed. HSC-derived T cells specifically trained to

recognize and kill tumors and virus-infected cells can be readily transplanted into patients. Currently, such treatments are not yet viable options, but preliminary successes have been demonstrated in treating human malignant melanoma, leukemia, a other types of cancer (Engelman, 2003, Galea-Lauri, 2002, Morse 2000, Schultz 2001).

Before delving into details, the following is a brief summary of the topics to be discussed. I will explain the process by which T-cells arise from hematopoietic stem cells (HSC) via the Notch signaling pathway. However, since HSCs are very difficult to maintain *in vitro*, we have selected other cell types as model systems for studying the Notch. The Notch pathway –which controls important developmental processes— consists of a series of proteins that work in conjunction to regulate important gene expression. I will then examine the current understanding of Notch's role in directing differentiation –or specialization of a cell-- from HSC to T-cells. This process of HSC differentiation to T cell has been difficult to reproduce in vitro with current cell-culturing methods. For this reason, the Dr. K. Roy proposed a new method utilizing magnetic beads that are coated, or functionalized, with protein to activate the Notch pathway. Our lab has demonstrated preliminary data for the proposed system's ability to direct the HSC to T cell differentiation process. My project was to visually demonstrate that the functionalized magnetic beads can activate the Notch pathway by staining the Notch receptor with fluorescent antibodies and to study the genes regulated by the Notch receptor. The intent is to improve the system by optimizing control of the Notch activation in the T cell developmental processes.

T cell development

For this paper, discussion will be restricted to the adult T cell development process, which begins with a particular type of HSC that migrate from the bone marrow to the thymus, an

organ located in the upper chest. There, the developmental progress of the HSC into T cells can be tracked with specific cell surface markers, with CD4 and CD8 as two of the most important markers. CD8+ cells (cells with CD8 glycoproteins on their surface) become killer or cytotoxic T cells (T_c), which destroy tumor and virus-infected cells. CD4+ cells become helper T cells (T_H), which are tremendously important to the adaptive immune system. T_H cells cannot kill invading pathogens, but they play the important role of activating and promoting the growth of T_c cells among other important functions. CD4+ are the target of the human immunodeficiency virus (HIV), and once HIV destroys enough CD4+ cells, the patient develops AIDS, a disease in which the immune system fails and the patient eventually die from a host of opportunistic diseases and tumors. A healthy immune system is imperative for an organism's survival, thus the importance in CD4+ and CD8+ T cells is apparent.

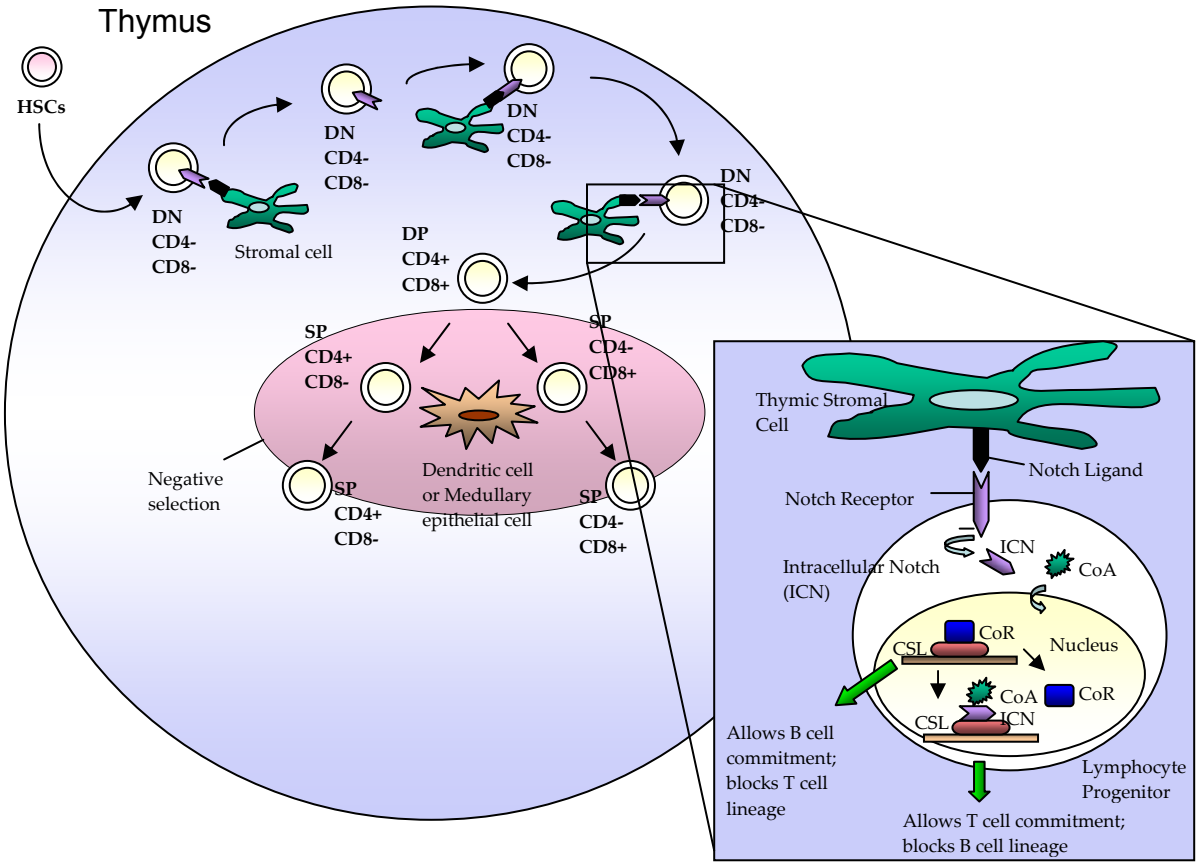


Fig. 1. Simplified T cell development in the thymus.
A particular type of HSC called lymphoid progenitor cells capable of generating T and B cells migrate from the bone marrow to the thymus. The differentiation process is partially guided by the thymic stromal cells that provide the signals necessary to differentiate HSCs into T cells. The progenitor cells begin as CD4 and CD8 double negative (DN) cells. In the next stage of differentiation, the progenitor cells become double positive (DP) before losing either CD4 or CD8 protein to become single positive (SP) cells that will become either T_c or T_H cells. Inset: Notch ligand on the thymic stromal cell surface binds the Notch receptor on the HSC, causing the intracellular portion of Notch (ICN) to break and move to the nucleus and act as either transcription enhancer or suppressor.
Modified from Zuniga-Pflucker, Juan Carlos. T-cell development made simple. *Nature Immunology* 4: 67-72, 2004

Referring to Figure 1 for T cell development, a subset of HSCs called lymphocyte precursor cells (can differentiate into the white blood cells types of B cells, natural killer cells, and T cells) originate in the bone marrow and then travel via blood to the thymus. In the thymus, HSCs interact with the microenvironment, receiving signals from the cytokines and ligands (molecules that bind to specific proteins) attached to the surface of stromal cells (loose connective tissue cells) that result in highly specific cell differentiation fate. Initially, HSCs are double negative for CD4 and CD8 surface proteins. In the next stage of differentiation, the precursors become double positive and undergo positive selection, where only cells expressing specific and functional surface markers are allowed to survive. Double positive cells will then become single positive, losing either CD4 or CD8 proteins to become T_H or T_C cells, respectively. At the single positive stage, the cells undergo a process by which T cells that attack the body's own cells are eliminated while others are released into the bloodstream as functional T cells that recognize abnormal or foreign (e.g. pathogen) cells.

In summary, the human body produces T cells vital to the immune system through a highly specific differentiation process, guided by signals and ligands from stromal cells in the microenvironment. This temporally and spatially orchestrated process occurs daily in the human body.

Notch signaling

One crucial component of the microenvironment in the thymus is the Notch signaling system. Notch genes are highly conserved (found with little variation) from invertebrates to mammals and play important roles in controlling cell fate in the generation of nerve, blood, and muscle cells as well as wing formation and eye development (Hozumi 2003). The Notch signaling system, which leads to HSC differentiation into the T cell lineage, consists of the following (Fig. 1 inset):

1. A protein called the Notch receptor on the signal receiving cell's surface. All four types of the Notch receptors (Notch1-4) are expressed on the cells in the thymus (Huang 2003, Harman 2003, Anderson 2001). We limited our studies to Notch1.
2. Notch ligands (a molecule that binds with a specific receptor) are attached to the signal presenting cell's surface, and the ligands include Jagged 1-2 and Delta-like ligands (DLL1-4). Both DLL1 and DLL4 are expressed by stromal cells in the thymus (Anderson 2001, Felli 1999, Maillard 2005). We limited our studies to DLL4.

Upon activation of the Notch1 by DLL4, the intracellular portion of Notch 1(ICN) is cleaved and translocated to the nucleus to activate or oppress certain gene expression in HSCs (Oksana 2001).

More importantly, Notch1 signaling is necessary and sufficient for T cell lineage commitment (Fig. 2). As mentioned above, a common lymphoid progenitor cell, which arose from the HSC, can give rise to both T and B cells. Numerous loss-of-function and gain-of-function studies have well established Notch1 as a key molecule that directs progenitor commitment to the T-cell lineage (Pui 1999, Wilson 2001, Artavanis-Tsakonas 1999). Notch1 activation instructs the HSC to become T cells, but in the absence of delta-ligand activation of

Notch1, the lymphoid progenitor becomes B cells by default (Zuniga-Pflucker 2004). In other words, overexpression of Notch1 leads to T cell development but block in B cell development while knock-outs of Notch1 lead to block in T cell development and irregular development of B cells in the thymus (Radtke 1999, Pui 1999, Kumano 2003, Izon 2001). (maybe find another word for block in the last sentence, too many blocks) Moreover, inactivation of Notch1 in vivo leads to a complete block of T cell development, implying other Notch receptors are not able to compensate for this loss of function (Radtke 1999).

However, the mechanism by which Notch1 leads to T cell lineage commitment is unclear. Few direct downstream target genes of Notch signaling have been identified, and of these I limited my studies to Hairy/Enhancer of Split 1 (HES1), a transcriptional repressor believed to aid in the survival and expansion of immature T cells (Midori 2000).

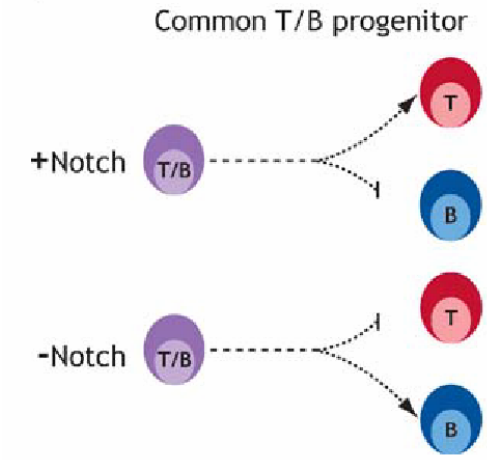


Fig. 2 Notch determines common lymphoid progenitor cell fate.
Notch signaling activation results in progenitor commitment to T cell lineage and a block in B cell development. Absence of Notch signaling results in progenitor commitment to B cell lineage and a block in T cell development.

From Maillard, I. et al. Regulation of lymphoid development, differentiation and function by the Notch pathway. *Annu. Rev. Immunol.* 23. 2005. 945-974

Drawbacks of current T cell production culture systems

Since Notch1 activation is necessary and sufficient for the HSC commitment to the T cell lineage, establishing control over the Notch signaling system would then allow control over the T cell differentiation process. Currently, the two major systems for generating T cells are the fetal thymus organ culture (FTOC) and stromal cell co-culture system. In FTOC, the thymus is

extracted intact, and its endogenous cells are washed out and then reconstituted with only the desired stromal cell types; progenitor cells are then added and allowed to differentiate (Yeoman 1993). However, this system is widely variable, complicated, cumbersome, and provides low T cell yield (Zuniga-Pflucker 2004).

The stromal cell co-culture system is more recent and provides a simpler HSC to T cell culturing method. This system utilizes OP9-DLL1 cells, which are OP9 stromal cells (loose connective tissue cells found in the bone marrow) that have been retrovirally transfected with the DLL1 gene, causing the OP9 cells to constitutively express DLL1 on the cell surface (Schmitt and Zuniga-Pflucker 2002). This allowed researchers to grow HSC and OP9-DLL1 cells together in tissue culture plates to induce the T cell development. Zuniga-Pflucker, a pioneer of this method, showed that OP9-DLL1 co-culture system could generate CD4+ CD8+ double positive cells and CD8+ single positive cells from HSCs (2004). Although the system is a great improvement to the FTOC, its design is not ideal for a large-scale production of T cells. First, separation of T cells from OP9-DLL1 cells in the mixed co-culture system would be cumbersome at large-scale; a transplantable T cell population should be as pure as possible. Second, it would be difficult to test other ligands of the Notch family since each ligand must be *stably* transfected into OP9. This process is time-consuming, disrupts normal gene expression in stromal cells, and risks the activation of proto-oncogene.

Moreover, neither of the systems afford significant control nor manipulation of the Notch ligand. One, removal of Notch signaling cannot be performed since OP9-DLL1 cells are in a mixed co-culture. Two, neither designs allow for temporal nor ligand-density control of DLL1 presentation to HSC. Several studies have shown that Notch signaling directs HSC to T cell differentiation in a density-dependent manner (Dallas 2005, Lehar 2002).

A new approach: synthetic Notch ligand substrate

The design by Roy lab addresses the limitations described above by presenting the delta-like ligand on magnetic microbeads and physically separating untransfected OP9 cells from HSCs.

Instead of using transfected OP9 cells to present the delta-like ligand, we use magnetic microbeads to present DLL4. We selected DLL4 because studies on this ligand has been limited even though DLL4 and DLL1 can both activate Notch1 and are both capable of inducing HSC commitment to T cell lineage (Hozumi 2004, Radtke 2004). The magnetic beads are 4.5 μm in diameter and surface-coated with streptavidin which strongly binds to the biotin on the antibody that binds DLL4 (see Fig. 3 for a more technical explanation). In this way, functionalized microbeads act as mimics of stromal cells that normally present delta-like ligand to the HSCs in the thymus. The added advantage is that we can quantify and adjust not only the amount of DLL4 on each bead but also the total amount of DLL4 added to a tissue culture by calculating the total number of beads. In this way, we gain control over the ligand density and now have a method to measure HSC response to varying doses of DLL4. Moreover, the beads can be added or removed as desired since the beads do not require careful growth conditions as do OP9-DLL1 cells, and beads can be readily removed with suction or magnets. The system provides unprecedented temporal and ligand density control that are not attainable in other systems.

To obtain a purer population of T cells, the HSCs and OP9 cells were physically separated by a permeable insert. The OP9 cells used are unaltered, meaning that they are not transfected to constitutively express DLL1. In this design, OP9 cells are seeded on the bottom of

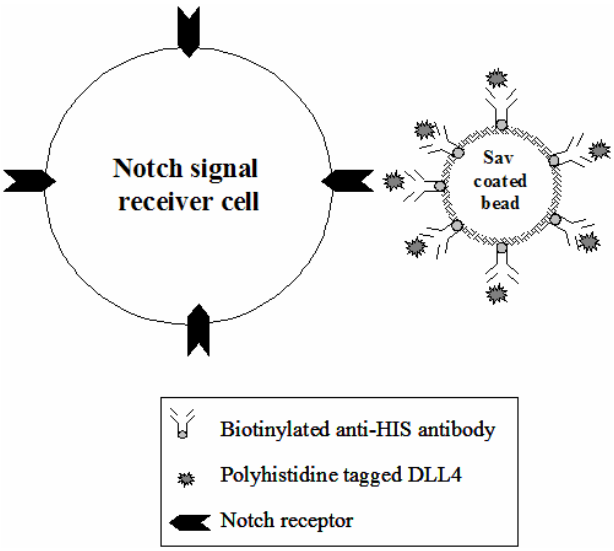


Fig. 3 Notch ligand-functionalized microbeads.
The noncovalent bond between streptavidin and biotin is one of the strongest, naturally occurring bonds. Taking advantage of this property, surfaces of magnetic microbeads are coated with Streptavidin which then binds the biotin attached to the anti-HIS antibody. The recombinant DLL4 with a HIS tag is then attached to the antibody. DLL4 is then immobilized and presented in a highly directional manner, allowing efficient binding and activation of Notch receptor on the signal receiving cell.
Image from Taqvi, S. *et al.* Biomaterial-based Notch signaling for the differentiation of hematopoietic stem cells into T cells. *J Biomed Mater Res A*. 79(3), 689-97 (2006).

tissue culture wells while HSCs are seeded in a permeable insert with DLL4 beads. The insert containing HSCs and beads are then placed on top of the OP9 cell culture plates so the two cell types share the same medium while remaining physically separated. Taqvi *et al.* showed that OP9 cells can support HSC differentiation through secreted and soluble paracrine factors that can cross the insert, so cell-cell contact is not required (2006).

Past studies have demonstrated the functionality of the DLL4 beads in providing Notch signaling to induce T cell lineage commitment. Bone marrow-derived HSCs in the insert co-culture system with DLL4 beads gave rise to T cells while HSCs cultured with uncoated beads did not, as measured by identifying T cell surface markers. Roy lab had shown that use of delta-

like ligand functionalized magnetic beads in combination with OP9 cells can direct HSC into the T cell lineage (Taqvi 2006).

Aims of this study

These previous bioactivity assays demonstrated that Notch signaling provided by DLL4 is necessary and sufficient for HSC commitment to the T cell lineage by examining the cell morphology and surface markers. The aim of my project is to characterize the cell-bead interaction to better understand the effects of quantitative and temporal manipulations of Notch signaling. Mouse cells were used since HSC development is best characterized in mice, and murine systems has served as models of human blood cell development (Tavian and Peault 2005). However, HSCs are notoriously difficult to expand and maintain in an undifferentiated state. HSCs isolated from mouse bone marrow are also very small in number, making large experiments very difficult. For these reasons, two other cell lines were used as model systems to test the dosage response to DLL4 beads. One is R1 murine embryonic stem (ES) cells, which are known to carry Notch1 receptors (Nemir 2006). The other cell line used is the C2C12 myoblast. Notch signaling system inhibits myoblast differentiation into myotubes, so this cell line is used as a classic assay for determining efficiency of Notch signaling (Lindsell 1995). These two cell lines were also selected since both are easily available and expand quickly.

My role is to study the DLL4 bead-cell interaction with respect to effects of bead-to-cell ratios and of varying bead-cell incubation time by using immunofluorescent staining (fluorescent staining of Notch1) and reverse transcriptase-PCR (method to detect gene expression). I expected to obtain visual confirmations of successful Notch signaling through staining the intracellular portion of Notch in both in C2C12 myoblasts and R1 cells. At the same I also wanted to find an optimum bead-to-cell ratio for Notch activation since previous studies showed

that utilizing 1:1 bead-to-cell ratio in the HSC/OP9 co-culture system yielded more HSC-derived cells expressing T cell markers while the 5:1 ratio yield almost no such cells (Taqvi 2006). I assessed the effect of different bead-to-cell ratios with IF staining, and Taqvi studied the same question with quantitative measures of gene expression (qRT-PCR).

MATERIALS AND METHODS

Conjugation of Notch ligand DLL4 to microbead

The microbeads used are Biotin Binder Kit's streptavidin-coated, superparamagnetic polystyrene microbeads from Dynalbiotech (Brown Deer, WI). First, washed microbeads were incubated with 1ug/ml of biotinylated, anti-6x HIS tag antibody (R&D Systems, Minneapolis, MN) for 30 minutes at room temperature. Next, beads were washed and incubated with HIS-tagged DLL4 protein (R&D Systems, Minneapolis, MN) at 2-4 ug/mL for 30 min at room temperature. Finally, beads were washed again and stored at 4°C. Detailed method for conjugating delta-like ligand 4 (DLL4) to magnetic microbeads can be found in previously published work (Maus 2002, 2003).

Cell culture:

Undifferentiated mouse R1 ESCs were a generous gift from A. Nagy, Mount Sinai Hospital, Ontario, Canada. R1 cells were expanded in the presence of leukemia inhibiting factor (LIF)-producing STO cells (irradiation-inactivated embryonic fibroblast cells from Shan Maika, Austin, UT) in complete DMEM medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (Hyclone, Logan, UT), 2 mm L-glutamine, 1 mm sodium pyruvate, 0.1 mm non-essential amino acids, 5×10^{-5} m 2-mercaptoethanol, penicillin G (100 U/mL), and streptomycin (10 μg/mL) (all from Invitrogen, Carlsbad, CA).

C2C12 cells (ATCC, Manassas, VA) were cultured and maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Logan, UT) and 2 m*M* L-glutamine (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2.

Immunofluorescence staining:

Cells were trypsanized from culture flask and seeded on 22 by 22 mm poly-L-lysine coated glass coverslips at 37°C. Seeding density is 100,000 cells/coverslip for R1 cells and 75,000 cells/coverslip for C2C12 cells. Cells were incubated overnight at 37°C with 5% CO_2 and incubated the next day with DLL4-functionalized beads in varying bead-to-cell ratio and incubation times (see result section for ratio and time). Uncoated beads were used as control. Cells were then fixed in 4% paraformaldehyde at room temperature for 20 minutes, washed with phosphate buffered saline (PBS), and then permeabilized with 0.25% Triton X-100 for 10 minutes are room temperature and washed with PBS. Samples were blocked in 1.5% goat serum in PBS for 1 hour, and antibodies were diluted 1:750 with 1% BSA, 0.05% Sodium azide in PBST. Cells were incubated overnight at 4°C with primary antibody rabbit anti-mouse/human activated Notch antibody (Abcam, Cambridge, MA), incubated 1 hour at room temperature with Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA), washed with PBST, and stained with DAPI. After mounting in Vectashield (Vector Laboratories), samples were imaged with the Zeiss Apotome Axiovert 200 microscope. Another secondary antibody tested was FITC-conjugated goat anti-rabbit antibody (Sigma-Aldrich, St. Louis, MO).

Bead staining:

Microbeads were blocked for 1 h at 4°C with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Microbeads were then stained with anti-mouse DLL4 antibody (R&D Systems, Minneapolis, MN) and FITC conjugated goat anti-rat IgG (eBioscience, San Diego, CA) for 1 hour at 4°C. Another secondary antibody used was APC-conjugated goat anti-rat (BD Pharmingen, San Jose, CA). Antibodies were diluted 1:750 with 3% BSA in PBS. Uncoated and biotinylated anti-6x HIS tag antibody coated microbeads served as controls.

RT-PCR:

Total RNA was isolated using methods previously described (Jakubowski and Roberts 1992, Gore and Roberts 1994). To remove genomic DNA, RNA was then treated with TURBO™ DNAse (Ambion, Foster City, CA) according to manufacturer's instructions. RNA concentration and purity were measured with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Reverse transcriptase-PCR: cDNA synthesized from mRNA transcribed with AccessQuick™ Master Mix (2X) and AMV reverse transcriptase (Promega, Madison, WI) according to manufacturer's instructions. PCR consisted of 40 cycles of 45 s at 94°C, 1 min at 60°C, and 1 min at 72°C. PCR products were separated by 2% agarose gel electrophoresis and were visualized with ethidium bromide staining. B-Actin served as housekeeping gene, and RNA from cells cultured with uncoated beads served as negative control. qRT-PCR: DNA contamination of RNA was assessed with a no reverse transcription control of all samples. cDNA samples were synthesized as previously described and analyzed in triplicates by SYBR green real-time PCR according to manufacturer's instructions (SuperArray, Frederick, MD). We selected B-Actin as the housekeeping gene. RNA extracted from myoblasts cultured with uncoated beads served as negative control and measurements were normalized to plain myoblast for any baseline Notch signaling. Data analysis was performed with SDS2.3 (Applied Biosystem, Foster City, CA) and Microsoft Excel. Hes1 and B-Actin primers for RT-PCR and qRT-PCR were ordered from SuperArray (SuperArray, Frederick, MD).

RESULTS:

DLL4 attachment to Bead.

In previous studies, Taqvi had demonstrated efficient functionalization of DLL4 to the microbeads and quantified the amount of DLL4 on beads with FACS and ELISA, respectively (2006). I then performed immunofluorescent staining to visualize the distribution of DLL4 on the functionalized beads to ensure an even coating of the bead surface. The expected image is a hollow center and a ring of fluorescence on the surface of the beads, indicating DLL4 coating. For all experiments, DLL4 and uncoated beads (control) were stained with anti-DLL4 primary antibodies raised in rats. FITC conjugated goat anti-rat secondary antibody then bind to the primary antibody. Another set of uncoated beads were incubated with only the secondary antibody as control. Optical section, fluorescent images taken with the 63X objective lens on the Zeiss Apotome Axiovert 200M microscope revealed that the polystyrene beads are autofluorescent in the FITC emission range. No significant differences were observed between images of control and variable group. All beads had an outer ring of FITC fluorescence due to autofluorescence and weak FITC signal in the center of the beads most likely due to noise from outside the depth-of-field range (Fig. 4). Other trials also indicated that the uncoated, unstained microbeads are also autofluorescent in the TRITC emission range, but not the DAPI emission range (data not shown). We then chose APC-conjugated secondary antibody to resolve the autofluorescence issue.

Since filters on the Zeiss Apotome microscope were limited to FITC, TRITC, and DAPI filters, we then utilized the Leica SP2 AOBS confocal microscope while keeping all other experimental conditions the same. Preliminary images indicated that the beads were not autofluorescent in the APC emission range (~660nm), and that a ring of APC fluorescence could

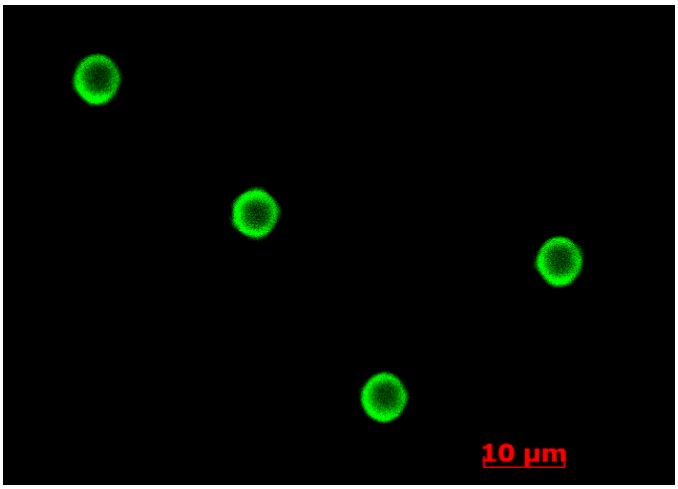


Fig. 4 Fluorescent image of uncoated beads with 63X objective lens. Uncoated and DLL4 coated beads were stained with anti-DLL4 primary antibody and FITC-conjugated secondary antibody. The polystyrene bead surface was autofluorescent and no significant differences were observed between unstained beads and stained DLL4 or uncoated beads. Weak FITC fluorescence in the center of beads is likely due to noise from outside the depth-of-field range.

be observed on the surface of DLL4 coated beads but not on the uncoated beads stained with either both antibodies or only the secondary antibody. However, clear images could not be obtained due to bead movements during the imaging process. Reducing the volume of liquid mounted the slides and reconstituting the beads entirely in VectaShield did minimize bead movement, but we were still unable to reduce movements enough to allow averaging of images to reduce background noise. A protocol to minimize bead movement was not developed due to time constraints.

Basal level of HES1 expression in R1 cells

In order to determine the basal level of Notch activation in R1 ES cells, a time course study was conducted with RT-PCR of HES1, a basic helix-loop-helix transcription factor and repressor shown to be a direct target downstream Notch signaling (Jarriault 1998, Kuroda 1998). R1 cells were allowed to proliferate and spontaneously differentiate in the tissue culture wells for 1 to 8 days, and total RNA was extracted at intervals of 24 hrs. RT-PCR of HES1 showed that basal level of HES1 expression persists even without the presence of DLL4 beads or other forms of added delta-like ligand to the tissue culture (Fig. 5).

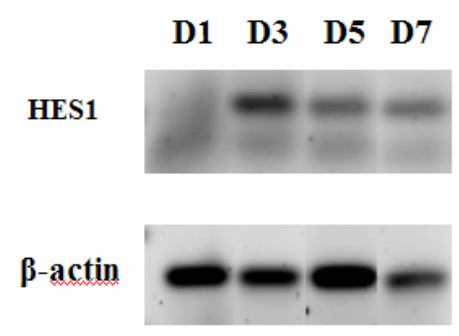


Fig. 5 Basal level of HES1 expression. Time course study of basal HES1 expression in R1 cells using RT-PCR. Figure shows RT-PCR data from total RNA extracted at days 1, 3, 5, and 7. 2 ug of RNA was used for cDNA synthesis. 100,000 ES cells were seeded in each well. N=3.

Basal level of Notch1 signaling in ES cells

Schmitt *et al.* detected higher level of HES1 expression in ES cells cultured with OP9-DLL1 cell than those cultured with OP9-control cells (2004). Since the DLL4 beads served as stromal cell mimics, I hypothesized that incubation with DLL4 beads would increase Notch1 activation in ES cells, and I wanted to assess ES cell's dosage response to different bead-to-cell ratios. In order to obtain a visual confirmation of Notch signaling activation in individual cells, I performed immunofluorescence staining for the intracellular portion of the Notch1 protein in R1 ES cells. After DLL4 activates Notch1, the intracellular portion of Notch1 (ICN) is cleaved and translocated to the nucleus. Cleaved ICN is then labeled with the primary anti-ICN1 antibody raised rabbit which binds FITC-conjugated goat anti-rabbit secondary antibody.

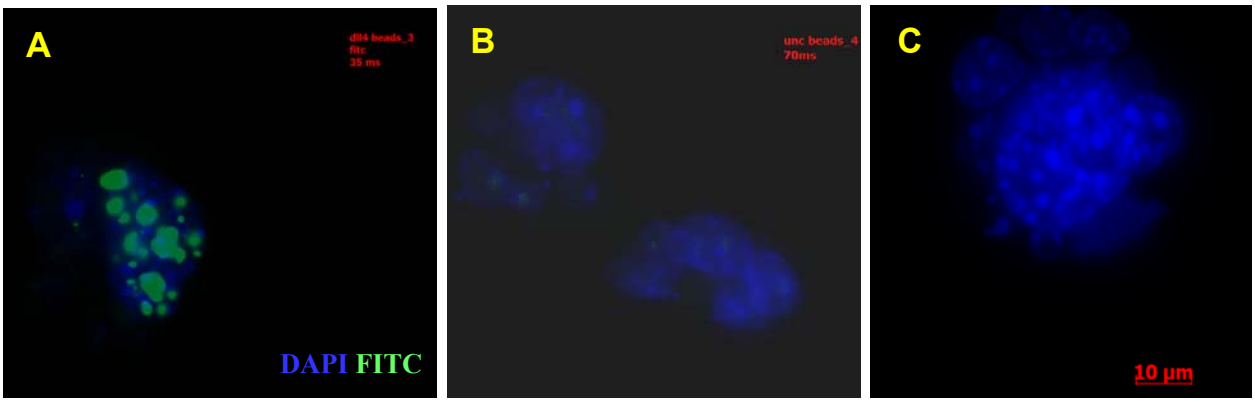
The methods for IF staining was first optimized through various trials to minimize non-specific fluorescent staining and to ensure reliability of the fluorescent images. Titrations of primary and secondary antibody include 1:100, 1:250, 1:500, and 1:750, and 1:750 dilution of both antibodies produced strong specific staining and minimal background. All R1 IF staining images were compared to that of the positive control SK-N-MC cells, which constitutively express Notch1. Therefore, 1:750 antibody dilution was used for all IF experiments. Moreover, we tested two types of secondary antibodies to minimize non-specific antibody binding. The

conditions tested were R1 cells incubated with no antibodies, secondary antibody, and both primary and secondary antibody. The control R1 cells with no antibody treatment were not fluorescent, indicating that R1 cells are not autofluorescent in the FITC and TRITC emission range. However, in R1 samples treated with only the secondary antibody, cells stained with the FITC-conjugated secondary antibody had high background, which could not be eliminated with increased blocking time, higher antibody dilution factor, or different blocking solutions (10% goat serum in PBST and 1.5% BSA in PBST). Invitrogen's Alexa Fluor 488-conjugated goat anti-rabbit antibody yielded less non-specific binding when used with Abcam's anti-mouse/human activated Notch antibody; thus this secondary antibody was used for all IF experiments.

First I assessed the effect of varying DLL4 bead incubation time with ES cells. ES cells were incubated either with no beads, DLL4 beads, or control beads (not coated with DLL4) for 15 min, 30 minutes, 1 hr, or 2 hrs. We selected human neuroepithelioma cell line SK-N-MC as the positive control since it constitutively expresses Notch1. Fluorescent images of ES cells in the three conditions did not exhibit significantly different levels of FITC fluorescence. Since the three incubation time had no observable effect on Notch signaling, we incubated cells with beads for 1 hour for all other experiments.

Previous data from Taqvi suggested that 1:1 bead-to-cell sufficiently transmitted Notch signaling (2006), so I attempted to obtain a visual confirmation of the Notch activation by the beads at this bead concentration. ES cells were incubated with either no beads, uncoated beads as control, or DLL4 beads. In only one experiment was I able to observe a difference in the activation of Notch receptors in ES cells cultured in the three different conditions (Fig. 6). ES

cell colonies incubated with DLL4 beads (Fig. 6A.) showed strong ICN staining while ES cells incubated with no beads (Fig. 6C) or uncoated beads (Fig. 6B) did not.



A 1:1 DLL4 bead to cell ratio **B** 1:1 uncoated bead to cell ratio **C** ES cell only

Fig. 6. Notch1 activation by DLL4 beads.
50000 R1 ES cells and STO cells were seeded on PLL coated coverslips overnight, incubated with DLL4 beads for 1 hr, and fixed and stained for the activated intracellular Notch domain with FITC. The controls were cells incubated with no beads (C) or uncoated beads (B). ES cells incubated with DLL4 beads (A) showed significant Notch1 activation while (B) and (C) had no detectable level of Notch1 activation.

However, I was not able to repeat this result in all other trials in which cells of all conditions exhibited level of fluorescence comparable to the ES cells incubated with no beads. Similarly, IF staining of ES cells cultured with bead-to-cell ratio of 0:1, 1:1, 5:1, and 10:1 yielded no significant different in fluorescence across all conditions (Fig. 7). The parameter and conditions tested in these experiments did not produce significant differences from the basal level of Notch signaling in ES cells as determined by fluorescence microscopy. Two possible explanations are (1) IF and fluorescent microscopy lack the resolution to distinguish changes in Notch1 activation that did occur with DLL4 bead incubation and/or (2) signaling from the DLL4 beads did not significantly alter basal level of Notch1 activity in undifferentiated ES cells.

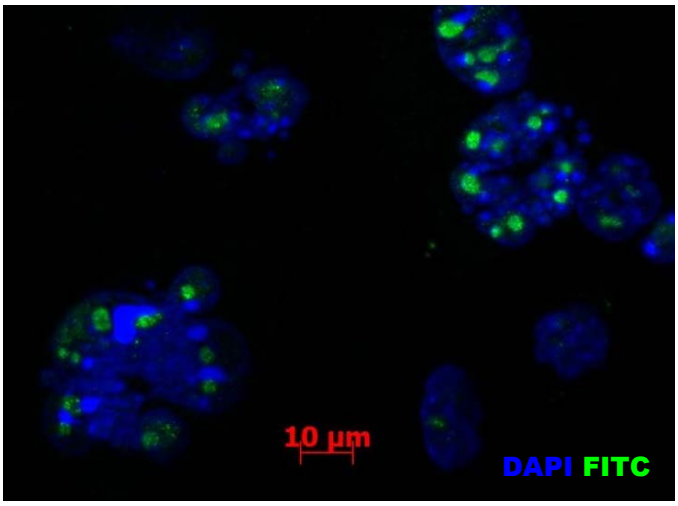


Fig. 7 Basal Notch1 activation in R1 cells.
R1 cells incubated with no beads showed strong FITC fluorescence, indicating basal Notch1 activation. Cells incubated with uncoated or DLL4 beads had similar level of fluorescence (not shown here). FITC represents the ICN and DAPI represents the nucleus.

Basal level of Notch signaling in C2C12 myoblasts

The classical myoblast assay demonstrates efficient Notch signaling by inhibiting the spontaneous myoblast to myotube differentiation. In past studies of classic Notch assay, Taqvi *et al.* demonstrated that the DLL4 beads were able to inhibit myotubes differentiation (2006). In order to examine the DLL4 beads' effects on the level of individual cells, I stained the cells with anti-ICN1 primary antibody raised in rabbit and FITC-conjugated goat anti-rabbit secondary antibody. C2C12 myoblasts were seeded on poly-L-lysine glass coverslips overnight and incubated with the conditions no beads, (control) uncoated beads, and DLL4 beads with bead-to-cell ratios of 1:1, 5:1, and 10:1. C2C12 staining results were similar to that of R1 cells. C2C12 myoblasts have a basal level of Notch signaling detectable by anti-ICN antibodies (fig. 8), and myoblasts cultured in all other conditions had Notch signaling levels that did not differ significantly from the basal level.

This led us to hypothesize that it is the decrease in basal Notch activation that allows myotube formation, thus Taqvi was able to inhibit the formation in the past study because the DLL4 beads sustained active Notch signaling, which blocked the gene expressions necessary for differentiation. In order to test this theory, we cultured C2C12 myoblasts on poly-L-lysine

coverslips with DLL4 beads for 6 days, and cultures with no beads or uncoated beads served as control. However, glass coverslips' poly-L-lysine coating was unable to maintain cell attachment for extended periods of time in cell culture. We were unable to optimize the protocol with the given time constraints, and it is also likely that IF staining cannot distinguish the changes in the Notch activity level with enough resolution..

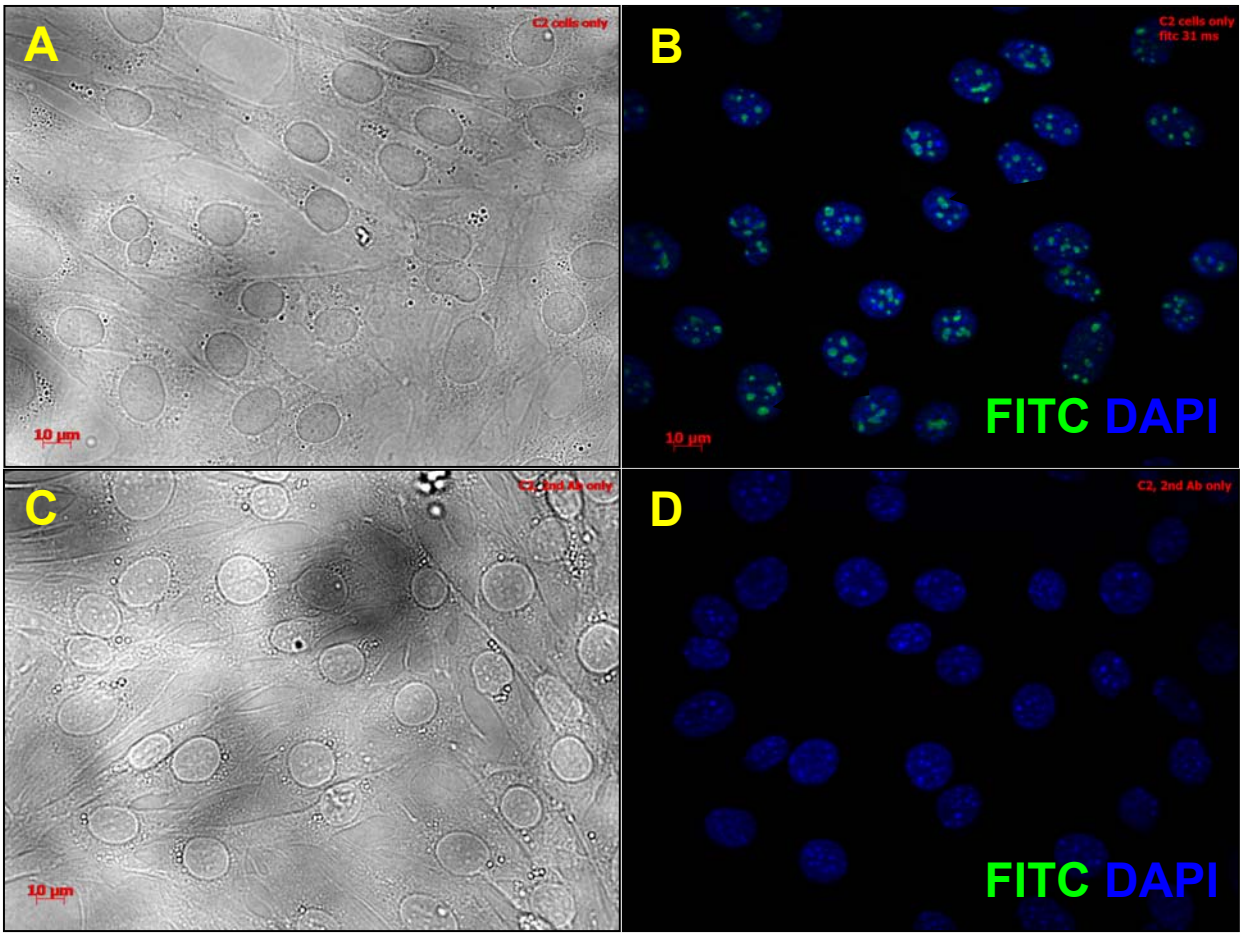


Fig. 8. IF staining of C2C12 myoblasts. A) Phase contrast image of C2C12 cells after 1 day of incubation with no beads. B) Anti-ICN fluorescent staining of A revealed basal level of Notch signaling even without presence of DLL4 beads. Myoblasts incubated with varying DLL4 bead-to cell ratios had similar level of Notch activity. C) Phase contrast image of C2C12 cells after 1 day of incubation with no beads. D) Fluorescent image of C stained with only the secondary FITC-conjugated goat anti-rabbit antibody illustrate that the ICN staining is specific and that C2C12 cells are not autofluorescent.

Secondary antibody	Bead-to-cell ratio	Dilutions	Cell-bead incubation time
Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen)	0:1	1:100	15 min
FITC goat anti-rabbit antibody (Sigma-Aldrich)	1:1	1:250	30 min
	5:1	1:500	**1 hr**
		1:750	2 hr

Table 1. Parameters tested for IF staining of R1 and C2C12 cells.
The parameters of the cell-bead interaction and IF staining include: secondary antibody, bead-to-cell ratio, dilution factor of both primary and secondary antibodies, and cell-bead incubation time. Conditions that yielded the best results are in bold.

Lower DLL4 bead-to-cell ratio increases Notch target gene expression.

Concurrently, Taqvi performed quantitative RT-PCR on Notch1 downstream target genes HES1. 100,000 C2C12 myoblasts were seeded per well for three days with either no beads or with bead-to-cell ratios of 0.1:1 and 1:1 with uncoated (control) or DLL4-coated beads. Measurements were normalized to cells cultured with no beads. Analysis indicates that HES1 expression was upregulated in myoblasts cultured with DLL4 beads. HES1 expression was ~5 fold higher in myoblasts cultured in 0.1:1 DLL4 beads than uncoated beads. Similarly, HES1 expression was ~4 fold higher in myoblasts cultured in 1:1 DLL4 beads than uncoated beads.

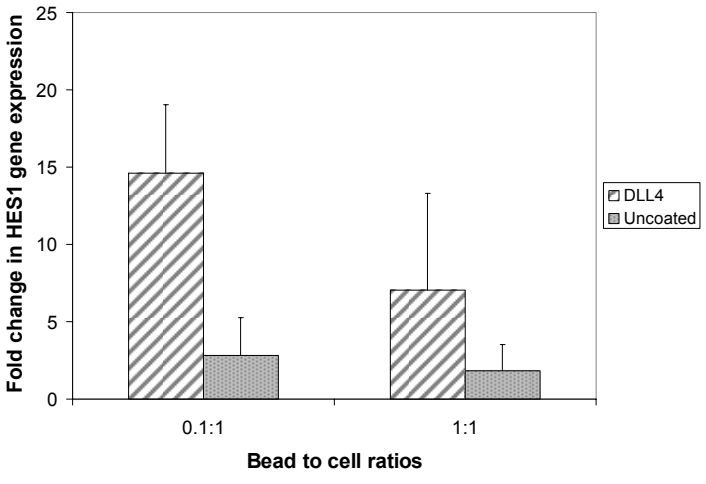


Fig. 9 qRT-PCR of HES1 gene expression in C2C12 cells.
HES1 gene expression was upregulated 4-5 fold in C2C12 myoblasts cultured with DLL4 beads compared to uncoated beads. Moreover, lower bead-to-cell ratio yielded higher increase in HES1 gene expression. β-Actin was the housekeeping gene, and N=3.

DISCUSSION

In summary, our lab has previously demonstrated the functionality of DLL4 beads in directing HSC to T cell lineage commitment. However, studies assessing the parameters of bead-cell interaction were limited. This synthetic Notch ligand substrate design is unique in that we use the beads for the derivation of T cells from stem cells while other groups have only used functionalized beads to activate already differentiated T-cells (Maus 2002 & 2003, Trickett 2002). My project aimed to characterize the bead-cell interaction to determine the effects of ligand density and duration of incubation on Notch1 activation by using two model cell lines murine R1 ES cells and C2C12 myoblasts. I attempted to obtain visualization of DLL4 distribution on the beads and to assess the aforementioned parameters to determine optimal utilization of beads for activation of Notch1 in HSC cultures.

The Notch signaling system has been established as a critical component of the T cell developmental process (Hozumi 2003, Schmitt 2004). In particular, Notch ligand activation of Notch1 receptors on lymphoid progenitors, a more specific type of hematopoietic stem cells, leads to cell fate commitment to T cells instead of B cells (Schmitt 2004, Maillard 2005). Hozumi demonstrated that both Delta-like ligands 1 and 4 (DLL1 and DLL4) are capable of inducing T cell commitment in vitro (2004). DLL1 and DLL4 share considerable homology and both are expressed in the thymus, and overexpression of DLL4 in HSCs blocks B cell development while promoting T cell development (Yan 2001). This led us to consider using DLL4 to create an efficient, high throughput biosynthetic system for producing T cells from HSCs. A system that can produce T cells "on demand" would be a giant leap towards making the idea of transplanting T cells into immunodeficient, cancer, and HIV patients a reality.

However, one major challenge in this field of research is that the Notch ligand must be

immobilized to activate its receptor (Varnum-Finney 2000). Current systems for T cell production utilize stromal cells with retrovirus-transfected genes to constitutive and exogenously express the Delta ligand. This includes the design by Zuniga-Pflucker using OP9-DL1 cells (2004). While these systems do allow for efficient HSC to T cell production, they all require a culture mixing stromal cells with HSCs, which makes it difficult to obtain a pure T cell population. Moreover, the process of obtaining a stable cell line transfected to express Notch ligand is cumbersome and the exogenous expression of the Notch ligand also disrupts normal gene expression (Lehar 2005).

The same drawbacks also exist in current biomaterial based systems. Poznansky *et al*. presented a system capable of generating human T cells in a biosynthetic matrix populated with murine thymic stromal cells (2000). These precedents are excellent tools for studying T cell development *ex vivo*, but they are be not viable for efficient, high level production of T cells due to mixed cell co-culture and lack of control over ligand presentation.

Our proposed system sought to address these issues by creating a system that allows high through-put production of T cells by using untransfected OP9 cells and DLL4-coated beads. OP9 cells and HSCs are physically separated while allowing OP9's paracrine factors to act upon HSCs. Moreover, presentation of Notch ligand on magnetic microbeads affords significant temporal and density control of ligand presentation. As previously mentioned, Taqvi had demonstrated that DLL4 beads are capable of committing HSCs to the T cell lineage. Nevertheless, a better understanding of the bead-cell interaction was needed.

First, immunofluorescent staining of DLL4-coated beads was done to visualize the distribution of DLL4 coating. A visualization of DLL4 distribution on the bead surface would help optimize DLL4 coating to ensure even distribution and therefore more controlled Notch

signaling activation when utilizing the DLL4-functionalized beads in cell culture. Previous studies showed that the bead coating method described above results in DLL4 coating of ~65% of beads and 130 ng of DLL4 conjugated to 1 million beads. As of now, we have not been able to obtain high resolution images of the DLL4 coating on beads due to the beads' autofluorescence. Nevertheless, we were able to be select allophycocyanin (APC) as the fluorophore (fluorescent molecule) that could be used to label the DLL4 without bead autofluorescence in the same excitation range. The last hurdle to imaging the beads is to minimize bead movement during confocal microscopy imaging. For future experiments, this may be accomplished by imaging the beads not on glass slides but in cell culture wells with glass bottoms. In this manner, movement of the microscope objective lens would not apply direct pressure on the beads, thus eliminating bead movement due to lens adjustments. Also, autofluorescence of beads in FITC and TRITC region are important factors to consider in future IF or FACS experiments

HSCs are difficult to expand and maintain in the undifferentiated state *in vitro*, so to assess the functionality of the DLL4 beads in Notch activation, we chose C2C12 myoblasts and R1 cells as model systems since Notch signaling plays important role in cell-fate determination in both cell lines.

To first establish the basal level of Notch activation in the R1 cells, reverse transcriptase-PCR was performed on HES1, a downstream target of Notch1 signaling, in R1 cells cultured with no beads from day 1 to day 8 (Jarriault, 1995, 1998). We found a low level of HES1 expression throughout the 8 days in culture without DLL4 beads. This is consistent with Schmitt *et al.* semi-quantitative RT-PCR findings from day 8 to 18, which showed low level HES1 expression in ES cells with no added Notch ligand (ESC-OP9 culture) and high level of HES1

expression in ES cells with added Notch ligand in the form of OP9-DLL1 cells (2004). Schmitt hypothesized that the low level HES1 expression activation in ESC-OP9 samples was due to OP9 expression of Jagged1 and Jagged2 (Schmitt 2004). We did not use OP9 for our experiments; however, undifferentiated ES cells are known to express the Notch ligands Jagged1, Jagged2, Delta3, and low levels of Delta1 (Lowell 2006). It is therefore likely that an ES cell's Notch1 receptors are activated by Notch ligands expressed on its own or other ES cells' surfaces.

Since Schmitt *et al.* was able to increase Notch1 activation with Notch ligands presented on stromal cells, I attempted to determine by IF staining if DLL4 beads could produce similar effects. IF staining was used instead of RT-PCR since the former allowed visual confirmation of Notch activation at the level of individual cells. However, if DLL4 beads did increase Notch signaling, fluorescent microscopy did not afford enough resolution to distinguish the variation. Varying culture conditions (duration of bead-cell incubation and varying bead-to-cell ratios with uncoated and DLL4 beads) yielded fluorescent images that did not differ significantly from ES cells cultured with no beads. As such, for purposes of demonstrating the functionality of the DLL4 beads in activating Notch1, perhaps the DLL4 beads' effect on ES cells would be more pronounced after ES were allowed to proliferate in culture for 12 days or more, the time point at which Schmitt *et al* demonstrated significant difference in HES1 expression between ES cells cultured with OP9-DLL1 and OP9-control (2004). Moreover, other studies have established that Notch1 is downregulated as ES cells begin to differentiate (Lowell 2006), so another possible experiment would be to examine if DLL4 beads can sustain Notch1 activity even as ES begin to differentiate. Due to current equipment and time limitations, we could not carry out the experiments described above, but they are nevertheless worthy of performing since a method to

visually evaluate Notch1 activity in ES cells would be a valuable tool to assess DLL4 bead functionality.

Similarly, repetition of abovementioned experiments with C2C12 cells yielded comparable results with no significant difference observed in images of IF staining for activated Notch1. Nevertheless, quantitative RT-PCR for HES1 revealed that cells cultured with DLL4 beads had a 4 to 5 fold increase in HES1 expression compared to the R1 cultured with uncoated beads. Also, the lower bead-to-cell ratio 0.1:1 led to higher HES1 expression than the 1:1 ratio. Therefore, IF staining should not be used to differentiate Notch activation level while basal activity is present in the cell. Previous studies demonstrated that 1:1 ratio resulted in higher T-cell differentiation than 5:1 ratio, and Taqvi demonstrated that 0.1:1 results in higher Notch1 activation than the 1:1 ratio. This has led us to hypothesize that higher bead concentration or DLL4 density has an inhibitory effect on Notch1 activation.

Future studies are needed to more accurately assess the dosage response of DLL4 functionalized beads on the Notch1 activation. To first obtain visual confirmation of Notch1 activation by DLL4 beads, IF staining should be performed on R1 ES cells cultured with DLL4 beads for more than 12 days and on C2C12 cells cultured with DLL4 beads after myotube formation begins in the control group. Staining at a longer time point will ensure that basal Notch1 activation has decreased enough that further Notch activation by DLL4 beads should be readily distinguishable by fluorescent imaging. These proposed studies would provide insight into how DLL4 beads alter Notch signaling in individual cells and into the pattern of Notch activation by the beads in the entire cell culture. Knowledge gained form the model cell lines can then be applied to optimize the HSC culture system. In addition, dosage effect of DLL4

beads on Notch1 activation should be assessed with quantitative RT-PCR to optimize the bead-to-cell ratio.

Although my studies were not able to confirm DLL4 bead activation of Notch via IF staining, the results will aid in developing the parameters for using murine ES cells and myoblasts as model systems to test the functionality of beads and bead-cell interaction. Knowledge gained from this study and the proposed future experiments will enhance our understanding of *in vitro* T cell generation. Moreover, continuation of the cell-bead interaction studies would further contribute to the development of a biomaterials-based system for high throughput T cell production for novel therapies.

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